

An Approach to Measuring Germinal Mutations in the Mouse

by L. R. Valcovic* and H. V. Malling*

Introduction

Recessive mutations with mildly deleterious effects are probably the most frequent class of mutations induced by environmental chemical mutagens. Crow (1) has suggested that in terms of long-range effects, these are potentially the most detrimental to the human population. However, there is a dearth of information on chemically induced gene mutations in mammals. This is due to the great difficulty in detecting such mutagenic events and, of course, the great expenditure of time and money required for mammalian genetic testing. Presently, the most efficient system for the detection of gene mutations is the specific locus method first devised by Russell (2) and later modified by Lyons and Morris (3). The method utilizes visible mutants with easily scorable phenotypes and; routinely, treated and untreated wild type mice are mated to the test stock. If a mutation occurs in the germ line of the treated individual at one of the specific loci of the test stock, the offspring will show the mutant phenotype.

The system can be applied to the study of germ cell stage sensitivity in either males or females. In relation to risk in the human population, the mutagenic sensitivity of male spermatogonia is the greatest importance since these are stem cells which multiply to produce sperm later; thus, a

mutation has a greater opportunity to be transmitted to offspring.

During the past twenty years the groups at Oak Ridge and Harwell have obtained a great deal of information on radiation-induced specific locus mutations. Of significance was the finding that the mouse is much more sensitive than *Drosophila* to ionizing radiation. Only a few chemicals have been tested in the specific locus method and hence a species comparison cannot be validly estimated. It is clear that some chemicals, notably TEM and PMS, are effective in causing specific locus mutations in mouse spermatogonia.

There are, however, disadvantages encountered in the specific locus test which, despite its simplicity, cause hesitation in recommending its use in a screening program. First, a significant proportion of the mutations are lethal when homozygous. In terms of a quantitative estimate of mutations rate, this is not detrimental; but with chemicals it is especially important to be able to study genetically and biochemically the induced mutations to determine the nature of the mutagenic event. Nonviable mutants may be due to the fact that the method detects only amorphic mutations; that is, either no protein is produced or the protein which is made is nonfunctional. This would result most often from small deletions or nonsense mutations. Only missense mutations involving highly critical amino acid sites would result in an amorphic mu-

*Mutagenesis Branch, National Institute of Environmental Health Sciences, National Institute of Health, Research Triangle Park, N. C. 27709.

tant. Hence, within the spectrum of gene mutation, those detected by the specific locus method are the most severe. Furthermore, because of the mutagenic specificity of chemicals, some may produce types of mutations which cannot be detected in the specific locus test. Second, in only a few loci is the specific protein involved in the mutation known, and thus the performance of the biochemical and molecular genetic procedures is extremely difficult. Even where the protein is known isolation of that protein is not easy. Among the radiation-induced mutants in Russell's laboratory there is a 35-fold difference in mutation rate within the seven loci studied. Without knowing the molecular aspects of these mutations, it is impossible to even guess about the basis for this large difference.

Third, since the method employs only seven loci, a very large number of offspring must be scored to detect a significant increase above the spontaneous rate. This factor is one of practical importance in consideration of cost of testing chemicals for mutagenic potential.

Electrophoretic Mobility Assay

Background

The assay system under development in our laboratory is based on a class of structural genes which code for enzymes which can be detected electrophoretically. These enzymes can be detected by specific histochemical staining after a crude tissue homogenate has been subjected to an electric field. Most commonly, starch gel or acrylamide gel are used as a supporting medium for electrophoresis. The technique is extremely simple since it requires only a crude homogenate yet is highly specific for particular enzymes because of the high degree of purity of commercially available substrates used in the histochemical identification. The term isozyme has been used to refer to the multiple molecular forms of an enzyme with common catalytic activities.

Electrophoresis is currently in wide use

to study natural genetic polymorphism in the area of population genetics. Since genetic polymorphism is based on the presence of two or more alleles at a given locus which have presumably arisen as a result of a past mutational event, it is merely a logical extension to use this approach as an assay for the detection of newly induced mutations. At the present time there are about 100 enzymes which can be detected by electrophoretic methods. The detection of mutants resulting in a change of the electrophoretic mobility is based on two requirements of the mutant enzymes: that they are enzymatically active and have a charge different from the wild type enzyme. The change in the charge of a protein can be the result of a base-pair substitution (BPS), or an insertion-deletion of one to several base-pairs of the DNA in a part of the gene not essential for catalytic activity, e.g., one-third of one end of an *E. coli* ribonuclease can be deleted without an appreciable change in its activity. A change in charge of an enzyme cannot be assumed to result from a base-pair substitution only. Certainly not all amino acid substitutions result in charge change; in fact, only about one-third of the genetically possible substitutions result in a charge change in the new amino acid. However, due to the three-dimensional folding of the primary chain to form the functional protein an amino acid charge change does not dictate a corresponding change in the total protein charge. Study of hemoglobin variants has supported this and further has provided examples in which there is a charge change in the protein but the amino acid substitution does not change the charge.

In addition to the EPM mutations it is desirable to detect inactive enzyme (IAE) or amorphic mutations. This can be accomplished by assaying the zymograms of heterozygous offspring from a cross between two inbred lines which differ in the electrophoretic pattern of several different enzymes. A simple example is in the case which the enzyme is a dimer; thus, the different parental strains each show only one band

that are of different mobilities. The heterozygous offspring shows three bands: the two parental bands and an intermediate band corresponding to the heteropolymer. A specific mutation in either parent can result in one of three different electrophoretic patterns: (a) the presence of only one of the two parental bands indicating a inactive enzyme mutation; (b) the presence of any two bands, one at the normal position for one of the parents, the other at any position indicating the inactive enzyme mutant will form active aggregate with the wild type enzyme; and (c) the presence of three bands, two with altered position, indicating and EPM-mutation. Since cases (a) and (b) indicate inactive enzymes and (c) and EPM enzyme, a direct estimation of the ratio of IAE-mutations and EPM-mutations can be made.

It is recognized that some mutations which do not alter the electrophoretic mobility of an enzyme do result in altered catalytic properties. One such type is that which increases the thermal lability of the enzyme. These mutations are usually detected by performing enzyme assays at a temperature which causes a slight inactivation of the normal enzyme and the mutant enzyme is greatly inactivated. Such heat-labile mutations can also be detected with the electrophoretic system by incubating the gel slice at the elevated temperature either just before or during the histochemical staining.

Protocol

Two strains of inbred mice have been chosen for our system, namely C57BL/6J and DBA/2J. These strains differ at nine electrophoretically detectable loci as shown in Table 1. Additionally, there are other loci which code for additional forms of some of these enzymes, e.g. esterase, phosphoglucosmutase and malic dehydrogenase. Since these other forms are identical in both strains, only EPM mutations will be easily scored. An IAE will result only in a slightly lighter band and this is difficult to see in starch gel electrophoresis. Also mutations at two

visible loci, d (dilute) and b (brown) will be detectable.

Horizontal starch gel block is the electrophoresis chosen for study. Sixteen samples can be applied in a single block which is 1 cm thick. After the electrophoretic run, the block is horizontally sliced into four slabs, each of which is stained for a different enzyme. However, since different enzymes may require different buffer systems for electrophoresis separation, it is not possible to use all four slices with the nine enzyme systems that are polymorphic. Therefore, we are incorporating other nonvariant enzymes to utilize all of the available gel slabs. The particular enzymes have not yet been selected but they will be selected from the list of enzymes that are currently being studied by Dr. James Neel, University of Michigan, Ann Arbor Michigan, in his program for monitoring the human population. Because this is a new method for detecting gene mutations and especially since very little is known about EPM mutations, it is important that the system be evaluated against a standard test system with a known mutagen. Therefore, our initial screen will be carried out by using ionizing radiation as the mutagen at a dose regimen of 500 R plus 500 R with a 24 hr interfraction period. Since this dose induces temporary sterility in males, we will assay only spermatogonia. The results from this experiment can then be compared with the specific locus test which has been so valuable in radiation genetics.

Table 1. Polymorphic loci presently available for point mutational assay.

Locus	Alleles		Tissue
	C57BL/6J	DBA/2J	
<i>Es-1</i>	a	b	Hemolyzate
<i>Es-3</i>	a	a	Kidney
<i>Gpd-1</i>	a	b	Kidney
<i>Gpi-1</i>	b	a	Hemolyzate
<i>Id-1</i>	a	b	Kidney
<i>Mod-1</i>	b	a	Kidney
<i>Pgm-1</i>	a	b	Hemolyzate
<i>Dip-1</i>	a	b	Kidney
<i>Hbb</i>	c	d	Hemolyzate

Adult males of each strain have been irradiated and will be mated to virgin females of the other strain. Tissue samples will be taken from mature F₁ individuals. One kidney will be surgically removed on the week 10, and a blood sample will be taken on the week 11. Electrophoresis will be run within 1 week from the time the tissue sample is obtained.

Color slides and/or photographs will be taken of each zymogram for accurate records of the results. Additionally, the color slides can be used for densitometric analysis to detect reduced enzyme activity mutants. Certainly the accuracy of this procedure will be dependent upon the range of normal enzyme activities and the precision in sampling and staining techniques.

Future System Development

As stated, there are presently nine protein differences between the two strains which can be detected electrophoretically. There are at least 13 other enzyme polymorphisms in other strains and stocks of mice. We have initiated procedures to have these other alleles placed into the C57BL/6J strain. Also, there are still many enzymes for which electrophoretic procedures are available that have not yet been applied to mouse tissues and we are planning to get these enzymes tested for polymorphism in mice. Thus, in about 5 years the system should have at least 22 loci which can be screened for both EPM and IAE mutations plus any new polymorphism.

Summary

A novel approach to the study of germ line mutations in the mouse has been described. The method is based upon standard electrophoretic techniques and established inbred mouse strains. The following advantages are cited.

1. Both electrophoretic mobility mutations

and inactive enzyme mutations can be detected in nine polymorphic loci. Also, EPM mutations can be scored in at least 10 additional nonvariant loci. Mutations which result in a thermal labile enzyme can also be detected in all loci.

2. Because the character under evaluation is an immediate gene product, biochemical techniques can be applied to determine the molecular nature of the mutational event. Procedures for purification of at least some of the proteins are established and subsequent peptide and sequence analysis are not beyond the scope of presently available techniques.

3. The increased number of loci in the system reduces the total number of progeny that need to be tested to detect an increase above the spontaneous mutation rate. Furthermore, the test alleles all have full catalytic activity; thus, they do not deter from the fitness of the individuals in the test, and the incorporation of additional variant alleles is not expected to decrease fertility. It is, therefore, feasible that as many as 50 loci could be tested in each individual. This not only will reduce the total sample needed but also will provide a more accurate evaluation of the variation in mutation rate among loci.

4. All of the test loci have comparable functions in man. Data obtained from these laboratory mammals will provide a baseline for the electrophoretic monitoring of the human population as proposed by Dr. James Neel. Screening chemicals for potential risk to the human population will also be more relevant in this system than any system presently available.

5. The majority of the enzymes in this system are dimers and hence are codominantly expressed. Therefore it would be possible to treat both parents in a single test and hence determine differential germ cell sensitivity in both sexes in a single experiment.

The primary disadvantage of the system is the time involved to test a single chem-

ical for mutagenic activity. However, if an *in vivo* mammalian assay is desirable then a minimum time of 3 months must be realized. A complete analysis with the present protocol will require about 1 year, but with an increase in the number of loci, it is conceivable that considerably shorter testing time will be achieved.

REFERENCES

1. Crow, J. F. The meaning of mutagenicity for society. EMS Newsletter No. 4: 22 (1971).
2. Russell, W. L. X-ray induced mutations in mice. Cold Spring Harbor Symp. Quant. Biol. 16: 327 (1951).
3. Lyon, M. F., and Morris, T. Mutation rates at a new set of specific loci in the mouse. Genet. Res. 7: 12 (1966).